An Autosomal Recessive Mutation of DSG4 Causes Monilethrix through the ER Stress Response

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Monilethrix is a hair shaft anomaly characterized by beaded hair with periodic changes in hair thickness. Mutations in the desmoglein 4 (*DSG4*) gene reportedly underlie the autosomal recessive form of the disease. However, the pathogenesis and cellular basis for the *DSG4* mutation–induced monilethrix remained largely unknown. We report a Japanese female patient with monilethrix. Observation of her hair shaft by means of transmission electron microscopy showed fewer desmosomes and abnormal keratinization. Genetic analysis revealed a homozygous mutation, c.2119delG (p.Asp707Ilefs*109), in the *DSG4* gene, which was predicted to cause a frameshift and premature termination in the intracellular region of the DSG4 protein. The mutation has not been reported previously. In the patient's hair shaft, we detected reduced but partial expression of the mutant DSG4 protein. Cellular analyses demonstrated that the mutant DSG4 lost its affinity to plakoglobin and accumulated in the endoplasmic reticulum (ER). The amounts of mutant DSG4 were increased by proteasome inhibitor treatment, and the expression of an ER chaperone, GRP78/BiP, was elevated in the patient's skin. Collectively, these results suggest that the dysfunctional mutated DSG4, tethered in the ER, undergoes ER-associated degradation, leading to unfolded protein response induction, and thus ER stress may have a role in the pathogenesis of monilethrix.

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INTRODUCTION

Desmosomes are adhesive intercellular junctions in the epidermis and myocardium and consist of several components, including desmoglein (DSG), desmocollin, and plakoglobin (PKG; Green and Simpson, 2007; Al-Jassar *et al.*, 2013; Ishida-Yamamoto and Igawa, 2014; Spindler and Waschke, 2014). DSG and desmocollin belong to the cadherin superfamily of transmembrane glycoproteins, and PKG, a member of the armadillo family, directly binds to the cytoplasmic tails of DSG and desmocollin. In humans, four *DSG* (*DSG1–DSG4*) genes have been identified on chromosome 18q12 (Whittock and Bower, 2003; Green and Simpson, 2007). DSG2 is ubiquitously expressed in desmosome-possessing tissues, including simple epithelia and the myocardium, whereas the expression of DSG1 and DSG3 is restricted to the stratified squamous epithelium (Schafer *et al.*, 1994). DSG4 is specifically expressed in the hair shaft cortex, cuticle, inner root sheath cuticle, and granular layer of the epidermis (Bazzi *et al.*, 2006).

Monilethrix, characterized by periodic beaded hair shafts and pronounced hair fragility, leads to hair loss. The autosomal dominant form of the disease (OMIM 158000) is caused by heterozygous mutations in basic hair keratin genes, such as *KRT81*, *KRT83,* and *KRT86* (Winter *et al.*, 1997; van Steensel *et al.*, 2005). Furthermore, *DSG4* mutations were found to underlie an autosomal recessive monilethrix (OMIM 607903) (Kljuic *et al.*, 2003). To date, only a few cases with *DSG4* mutations have been reported (Kljuic *et al.*, 2003; Meyer *et al.*, 2004; Moss *et al.*, 2004; Rafiq *et al.*, 2004; Schaffer *et al.*, 2006; Shimomura *et al.*, 2006; Zlotogorski *et al.*, 2006; Farooq *et al.*, 2011). Although a previous transmission electron microscopy (TEM) study on hereditary monilethrix suggested that hair shaft thinning was induced by the degeneration of hair cortical matrix cells (Ito *et al.*, 1990), the molecular basis for monilethrix, such as the identification of mutation sites and responsible proteins, was not revealed. Therefore, the clinical examination and the cellular basis for monilethrix caused by the human *DSG4* mutation have not been fully investigated.

Eukaryotic cells possess quality control mechanisms in the endoplasmic reticulum (ER) to monitor the folding of nascent

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Abbreviations: DSG, desmoglein; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum–associated degradation; PKG, plakoglobin; TEM, transmission electron microscopy; UPR, unfolded protein response; Wt, wild type

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secretory proteins. The accumulation of unfolded or misfolded proteins in the ER induces the unfolded protein response (UPR; Mori, 2000). In the course of UPR, unfolded or misfolded proteins initially induce the transcription of ER chaperones, such as GRP78/BiP and GRP94, to refold the misfolded proteins. Next, to reduce the amounts of misfolded proteins, the accumulated unfolded proteins in the ER are transported to the cytoplasm and degraded by the ubiquitin-proteasome machinery by ER-associated degradation (ERAD). Finally, the damaged cells undergo apoptosis for clearance. As DSG is a transmembrane-type glycoprotein and initially synthesized in the ER, the mutant DSG may be retained in the ER through the quality control mechanism.

Here, we report a Japanese patient with autosomal recessive monilethrix caused by a homozygous *DSG4* mutation, which encodes a frameshift mutation and a truncated cytoplasmic region of DSG4 with the addition of 109 aberrant amino acids. Some of the mutant DSG4 protein was detected in the patient's hair, but its affinity to PKG was markedly reduced. Furthermore, the mutant DSG4 was predominantly localized in the ER and degraded through the ERAD pathway. The accumulation of mutant DSG4 also induced UPR in both cultured cells and the patient's skin. These results suggested that the ER stress caused by the mutant DSG4 may have a role in the pathogenesis of monilethrix.

RESULTS

Clinical features

The patient was a 25-year-old Japanese woman (Figure 1a). Although we could not examine the father because of social reasons, the patient belonged to a nonconsanguineous pedigree in the known range, and the parents, in addition to her sister, were unaffected and had straight, black scalp hair with normal density. The patient had sparse and fragile scalp hair since birth (Figure 1b). Her eyebrows and eyelashes were also sparse, and there were numerous keratotic follicular papules with erythema on the scalp skin. In addition, she had dry skin and keratosis pilaris–like papules on the trunk and extremities (Supplementary Figure S1A online). A broken and buried hair was observed on the right arm by dermoscopy (Supplementary Figure S1B online). Histopathologically, the keratotic follicular papule revealed slight focal hyperkeratosis and a subcorneal curled and buried hair (Supplementary Figure S1C online). Her axillary hair and pubic hair were almost normal. The patient showed normal facial appearance, teeth, nails, and sweating. Some of the hair was buried in the horny layer (Figure 1c), which suggests the difficulty of hair penetrance to the skin surface. We confirmed a few strands of moniliform hair on the scalp. Dermoscopic evaluation revealed that the moniliform hair had black nodes and white internodes (Figure 1d). Periodic nodes and internodes were seen in the plucked moniliform hair by light microscopy (Figure 1e). Hematoxylin–eosin staining of her hair follicles revealed moniliform hair, with periodic nodes and internodes in the follicles (Figure 1f).

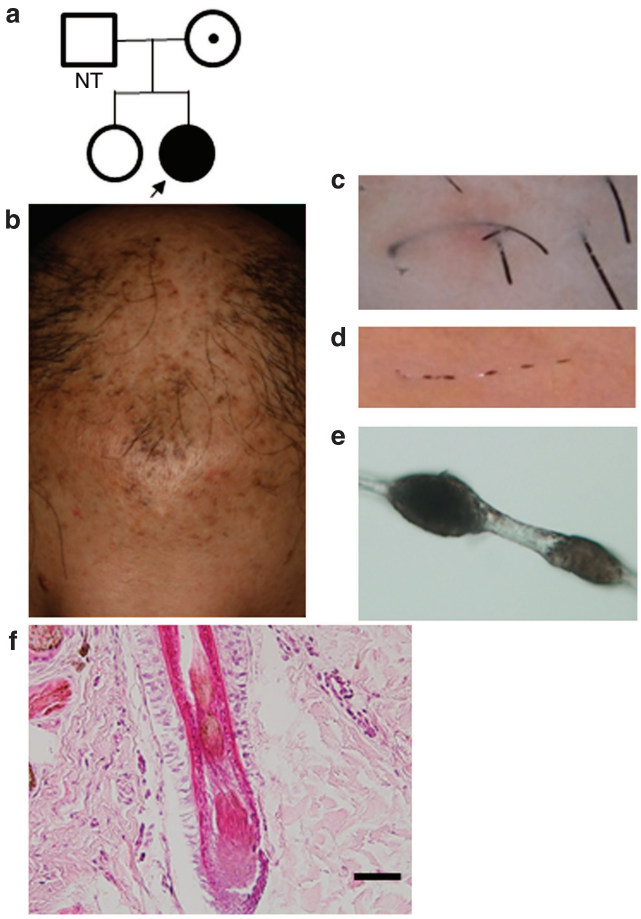

Figure 1. Clinical appearance of the patient. (**a**) The pedigree of the patient's family. The patient's mother was a carrier and her sister was normal. (**b**) Sparse hair and follicular papules on the scalp. (**c**) Dermoscopic examination revealed some bent and buried hair in the stratum corneum. (**d**) A typical moniliform hair on the patient's scalp, observed by dermoscopy. (**e**) Periodic nodes and internodes were seen in the plucked moniliform hair by means of light microscopy. (**f**) Hematoxylin–eosin stain of a moniliform hair in the lower hair follicles. NT, not tested. Bar = 100 μm.

TEM analysis of moniliform and anagen hairs

Light microscopy of semithin sections of the moniliform hair showed that part of the hair shaft was constricted (Figure 2a), in contrast with the straight outline of normal control hair (Supplementary Figure S2A online). TEM analysis of the constricted part revealed a dyskeratotic and discontinuous cortex as well as a dyskeratotic and distorted cuticle (Figure 2b). Intracellular granules were visible in the outer layers of the cuticle. The inner layers of the cuticle were dyskeratotic, with an electron-dense cytoplasm (Figure 2c and d, Supplementary Figure S2B and S2C online). Moreover, cytological abnormalities of cortical cells, such as thickened keratin fibrils and some unknown granular deposits, were observed (Figure 2e and f, Supplementary Figure S2D and S2E online).

A semithin section of the patient's hair revealed a broken anagen hair shaft (Figure 2g), in contrast with the normal appearance of control hair (Supplementary Figure S2F online). There were clear spaces in the cytoplasm among the keratin fibers (Figure 2h and Supplementary Figure S2G online). The cuticle cells of the patient's hair were distorted (Figure 2i and Supplementary Figure S2H online). Fewer desmosomes were

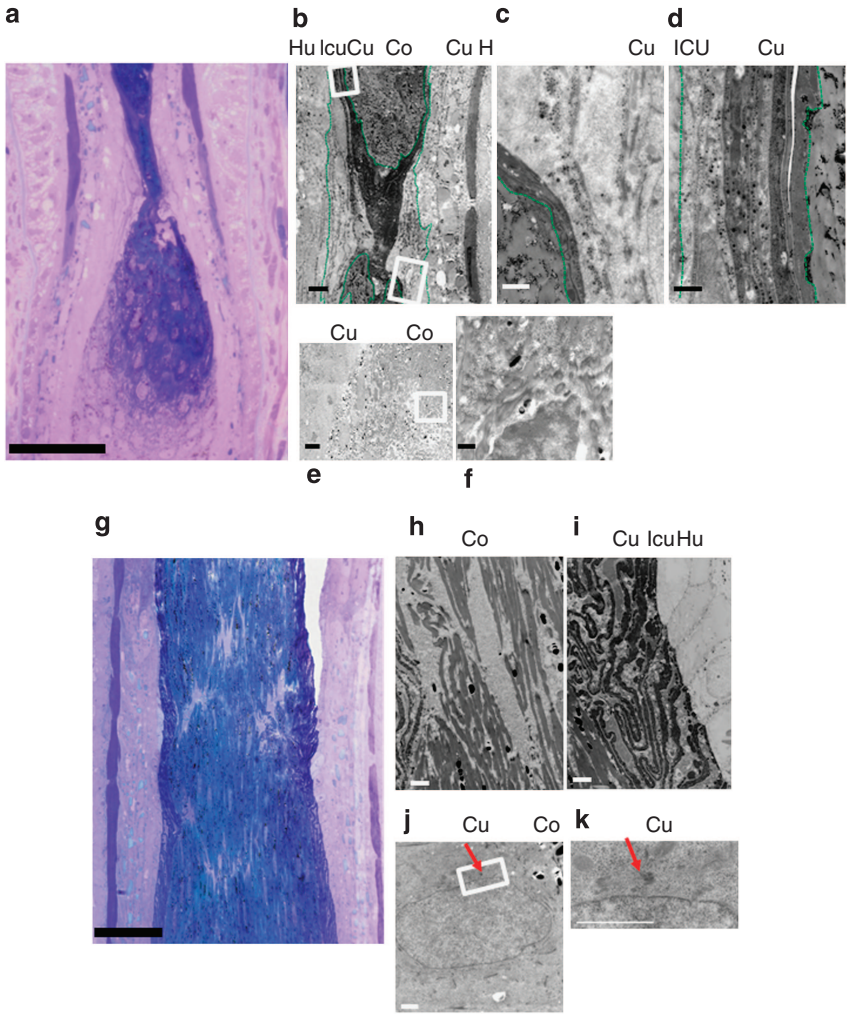

Figure 2. Transmission electron microscopy (TEM) analysis of a moniliform hair and an anagen hair of the patient. (**a**) Semithin section of a moniliform hair. (**b**) Dotted green lines: cuticle cells. (**c**, **d**) Higher magnifications of the bottom (**c**) and top (**d**) rectangles in (**b**). (**e**, **f**) The lower portion of the hair shaft. (**f**) Higher magnification of (**e**). (**g**) Semithin sections of the anagen hair. (**h**, **i**) TEM of the cortex (**h**) and cuticle (**i**). (**j**, **k**) TEM of cuticle cells in the lower hair follicle. Red arrows: desmosomes. (**k**) Higher magnification of (**j**). Co, cortex; Cu, cuticle; Hu, Huxley layer; H, Henle layer; Icu, inner root sheath cuticle. Bars = (**a**, **g**) 50 μm, (**b**, **e**) 5 μm, (**h**, **i**) 2 μm, and (**c**, **d**, **f**, **j**, **k**) 1 μm.

detected in the patient's cuticle cells, as compared with normal control cells (Figure 2j and k, Supplementary figure S2I–S2K online).

Identification of a mutation in the *DSG4* gene and its expression in the patient's hair follicle

On the basis of the clinical features, we diagnosed our patient as having monilethrix and performed direct sequencing analyses of the *DSG4* genes and other candidate genes. The mutation was a homozygous deletion of G at nucleotide position 2,119 in exon 14 of the *DSG4* gene (c.2119delG; Figure 3a). The mutation was not detected in 50 unrelated healthy control individuals (100 chromosomes) of Japanese origin (data not shown). We further performed direct sequencing of the exon–intron boundaries of the *KRT81*, *KRT83,* and *KRT86* genes; however, no mutations were detected (data not shown). The patient's mother carries a heterozygous mutation (Figure 3a) and her sister was normal (data not shown). The mutation, located in the intracellular anchor domain of DSG4, results in a frameshift and prematurely terminated protein fused with 109 aberrant amino acid residues (p.Asp707Ilefs*109; Figure 3b and c).

To clarify the expression of mutant DSG4, immunohistochemistry using a polyclonal anti-DSG4 antibody, which recognizes the extracellular domain, was performed. The DSG4 staining in the hair shaft cortex of the patient was much weaker than that of the control (Figure 3d and e). Therefore, to detect the mutant DSG4 in the patient's hair, we raised an antibody against the mutant DSG4–specific peptide, KSTPTPMQPGARWKE (Figure 3c). The antibody specifically detected the expression of mutant DSG4 in HEK293T cells (Supplementary Figure S4 online). Moreover, the patient's cortex and cuticle clearly reacted with the mutant DSG4–specific antibody (Figure 3f and g). To further confirm the

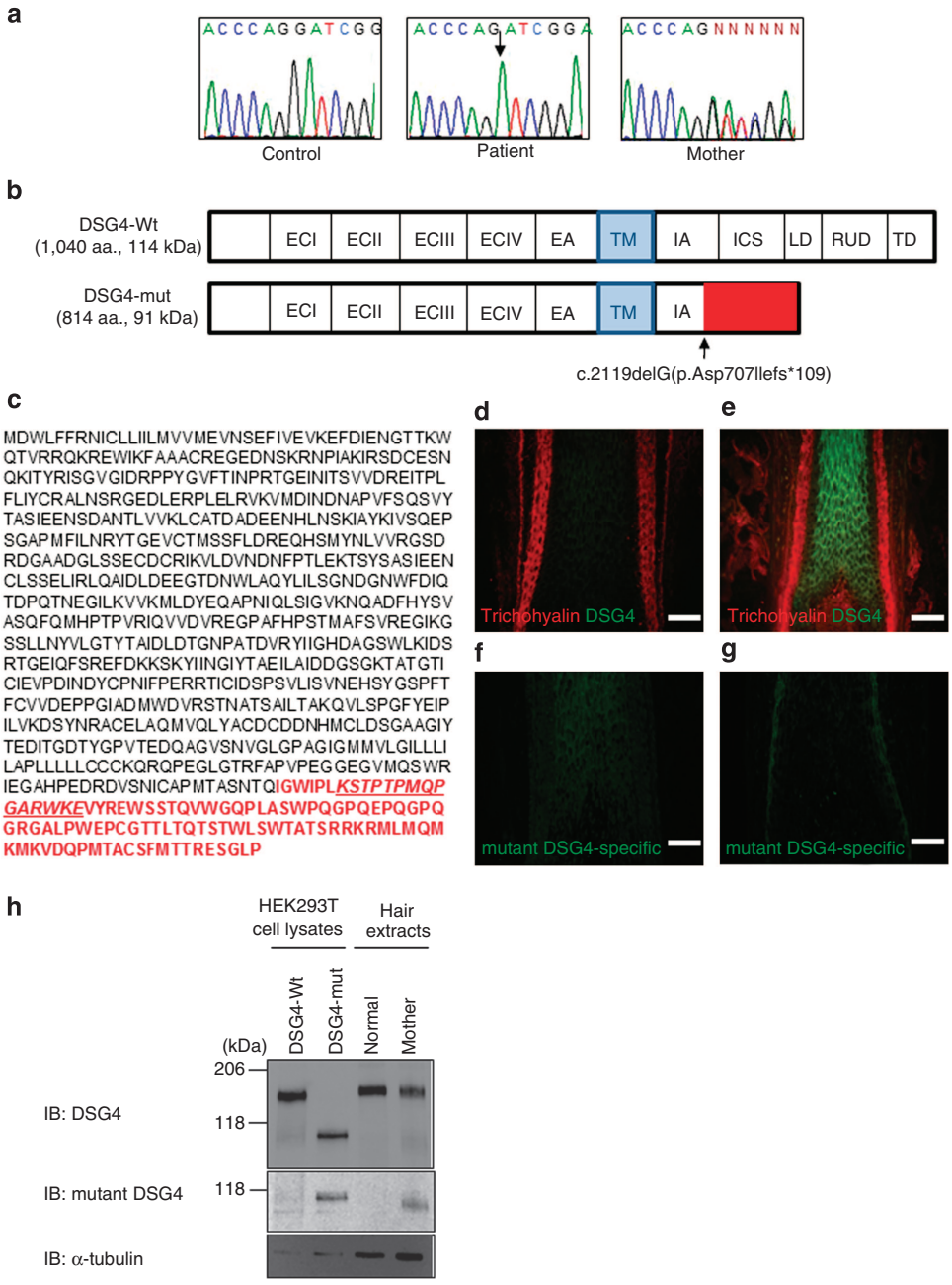

Figure 3. Identification of mutant DSG4. (**a**) Identification of the *DSG4* mutation. Arrow: the deleted G. (**b**) Domain organization of Wt and mutant DSG4. Arrow: the mutation site; red box: the aberrant region of the mutant DSG4. (**c**) Sequence of the mutant DSG4. Red: the aberrant sequence; underlined: the peptide sequence for the mutant DSG4–specific antibody. (**d**–**g**) Immunofluorescence of the hair cortex with anti-DSG4 (green) and anti-trichohyalin (red) antibodies (**d**, **e**) or the mutant DSG4–specific antibody (**f**, **g**). (**d**, **f**): Patient. (**e**, **g**): Normal. Bars = (**d**–**g**) 100 μm. (**h**) Immunoblotting to detect aberrant DSG4 was performed using the described antibodies. DSG, desmoglein; EA, extracellular anchoring; EC, extracellular; IA, intracellular anchor; ICS, intracellular cadherin-typical segment; LD, linker domain; RUD, repeat unit domain; TD, terminal domain; and TM, transmembrane.

expression of the mutant DSG4, immunoblotting analyses using polyclonal anti-DSG4 and mutant DSG4–specific antibodies were performed, using the mother's hair (Figure 3h). We attempted to examine the level of DSG4 in the patient's hair, but were unsuccessful probably because of the fragility of the hair and the loss of the follicle. We identified the expression of a faint but detectable amount of aberrant DSG4, and a slight reduction in the level of normal DSG4 in the mother's hair.

Aberrant mRNAs including a premature termination codon are reportedly degraded through the nonsense-mediated mRNA decay pathway (Maquat, 2005). We detected the premature termination codon of *DSG4* in the last exon, suggesting the production of an almost full-length mRNA.

Since the patient's hair was unavailable, we analyzed the stability of the *DSG4* transcripts expressed in her mother's hair. The reverse transcriptase–PCR analysis revealed the normal level of *DSG4* mRNA in her mother's hair (Supplementary Figure S3A online), suggesting that the mutant *DSG4* transcripts were stable and escaped from nonsense-mediated mRNA decay. Moreover, we performed PCR to amplify the spanning exons 13 and 14, which are located close to the poly A-tail, using reverse transcripts from a normal control and the mother, and then digested with *BspP*I, which can cleave the PCR product from the normal allele (Supplementary Figure S3B online). Although almost the entire amount of the PCR product from the normal allele was cleaved, a *BspP*I-resistant fragment was observed in the mother's PCR products (Supplementary Figure S3C online). These results suggested that the mutant DSG4 mRNA can escape from nonsense-mediated mRNA decay and is partially expressed in the patient's hair, although the amounts of mutant DSG4 were lower than that of wild-type (Wt) DSG4, suggesting the impaired trafficking of the mutant DSG4 protein.

The mutant DSG4 lacks affinity to PKG

In desmosomes, DSGs associate with PKG, and the intracellular cadherin-typical segment domain of DSG4 has a crucial role in binding PKG (Figure 3b; Mathur *et al.*, 1994; Troyanovsky *et al.*, 1994; Farooq *et al.*, 2011). As the patient carries an intracellular anchor domain mutation of *DSG4* and lost the normal intracellular cadherin-typical segment domain, the binding of mutant DSG4 to PKG was presumably affected. Indeed, coimmunoprecipitation analyses between the DSG4 and PKG proteins in HEK293T cells indicated that the mutant DSG4 protein showed markedly reduced affinity to PKG, although DSG4-Wt efficiently bound to PKG (Figure 4). These results indicated that the mutant DSG4 is dysfunctional and fails to bind PKG.

Mutant DSG4 is retained in the ER

To investigate the cellular basis for the mutant DSG4, we transiently transfected Wt and mutant *DSG4* in HEK293T cells, and monitored the cells by immunofluorescent staining. The mutant DSG4 protein was localized in the reticular organelle throughout the cytoplasm, but not in the nucleus (Figure 5a). These patterns were identical to those of calnexin, an ER-resident molecular chaperone (Figure 5c and e). On the other hand, the DSG4-Wt was predominantly expressed at the plasma membrane (Figure 5b) but not in the ER (Figures 5d and f). These results suggested that the mutant DSG4 was not transported to the cell surface but remained in the ER. To further confirm the intracellular localization of the mutant DSG4, we extracted the Wt and mutant DSG4 proteins and performed endoglycosidase H (Endo H) and PNGase F digestions. endoglycosidase H specifically cleaves high mannose–type *N*-linked oligosaccharides from glycoproteins, which are predominantly modified in the ER, whereas PNGase F can remove any type of *N*-linked oligosaccharide. The molecular weight of the mutant DSG4 was decreased after endoglycosidase H digestion, although DSG4-Wt was insensitive (Figure 5g). Moreover, faster migration of both the Wt and mutant DSG4 proteins was detected after PNGase F digestion. Collectively, these results suggested that the majority of the mutant DSG4 is retained in the ER.

Mutant DSG4 induces ER stress

Misfolded protein accumulation in the ER induces UPR, and typical ER chaperones, such as GRP78/BiP, are markedly induced through the response (Mori, 2000). We performed immunostaining of the patient's epidermis with an anti-GRP78/BiP antibody because of the limited availability of the patient's follicles. In contrast to normal control, the granular layer of the patient's epidermis was strongly positive for GRP78/BiP (Figure 6a and b).

Misfolded and tethered proteins in the ER are subjected to ubiquitin-proteasomal degradation after retrograde translocation to the cytoplasm (Bonifacino and Weissman, 1998). To investigate whether the mutant DSG4 is degraded through the ERAD pathway, we examined the DSG4 protein levels in the presence or absence of the proteasomal inhibitor, MG132. Compared with control cells, the intracellular amount of mutant DSG4 was increased by the MG132 treatment, whereas that of DSG4-Wt was not affected (Figure 6c), suggesting that some of the mutant DSG4 is proteasomally degraded through the ERAD pathway.

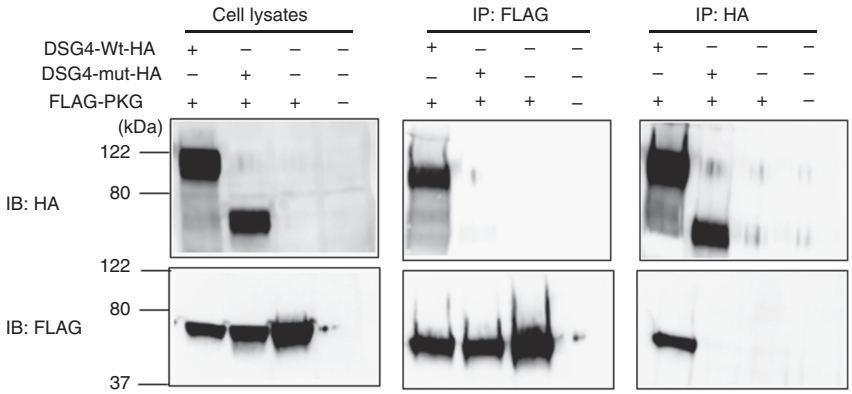

Figure 4. The mutant DSG4 failed to bind PKG. Expression plasmids, including pcDNA3.1-DSG4-Wt-HA, pcDNA3.1-DSG4-mutant (mut)-HA, and pcDNA3.1-FLAG-PKG, were transiently transfected into HEK293T cells. Cell lysates were prepared, immunoprecipitated with the indicated antibodies, and electrophoresed. Immunoblottings were performed with the indicated antibodies. DSG, desmoglein; IP, immunoprecipitation.

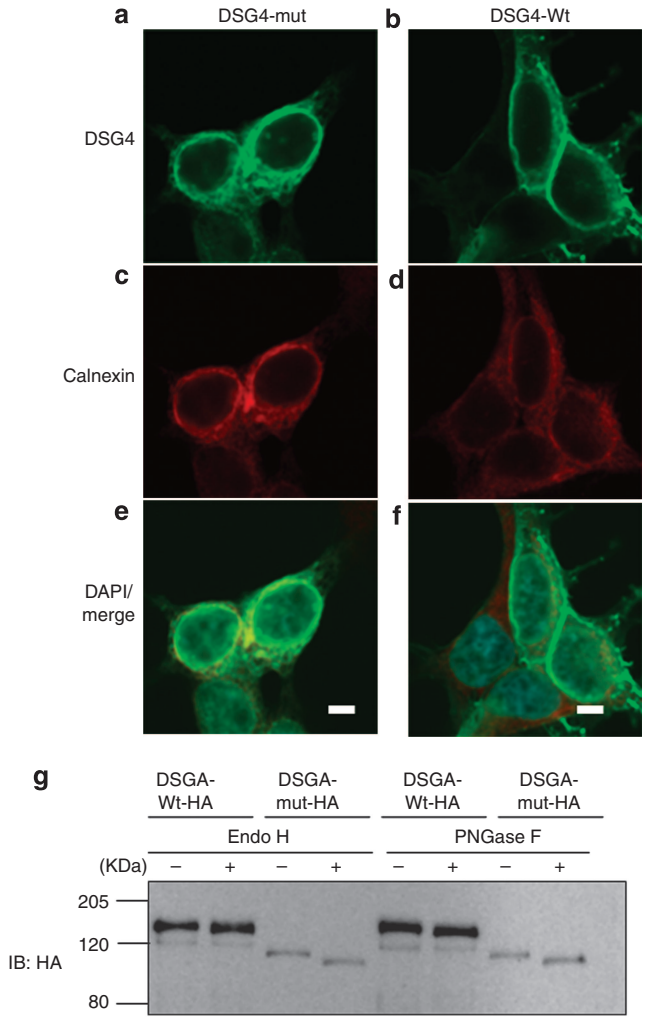

Figure 5. The mutant DSG4 is localized in the ER. Immunofluorescent staining of DSG4 and the ER-resident chaperone, calnexin. DSG4-mut-HA and DSG4-Wt-HA were expressed in HEK293T cells, and DSG4 (**a**, **b**) and endogenous calnexin (**c**, **d**) were stained. (**e**, **f**) DAPI stain and merged image. Bars = (**a**–**f**) 5 μm. (**g**) *N*-glycosylation of DSG4-Wt and DSG4-mut. The HEK293T cell lysates, from cells transiently expressing DSG4-Wt-HA or DSG4-mut-HA, were treated with Endo H or PNGase F. After SDS-PAGE, immunoblotting was performed with an anti-HA antibody. DSG, desmoglein; Endo H, endoglycosidase H; ER, endoplasmic reticulum.

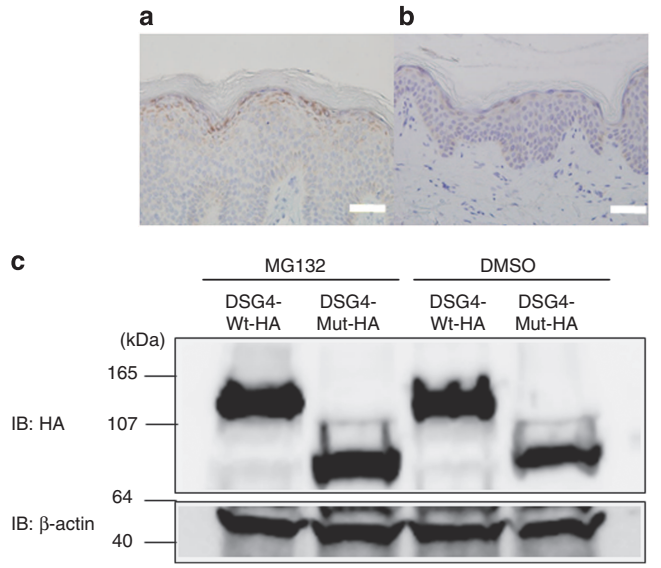

Figure 6. The mutant DSG4 tethered in the ER induces UPR and ERAD. Immunohistochemistry of the patient's epidermis, using an anti-GRP78/BiP antibody. In comparison with the normal epidermis (**b**), GRP78/BiP was slightly expressed in the patient's granular layer of the epidermis (**a**). Bars = (**a**, **b**) 100 μm. (**c**) HEK293T cells, transfected with pcDNA3.1-DSG4-Wt-HA or pcDNA3.1-DSG4-mut-HA, were treated with 10 μM MG132 or DMSO for 4 hours. The DSG4 proteins were detected by an anti-HA antibody. As an internal control, β-actin was also detected. DSG, desmoglein; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum–associated degradation; UPR, unfolded protein response.

These results suggest that UPR and ERAD are involved in processing the mutant DSG4 through the quality control mechanism in the ER. Taken together, the impaired trafficking and retardation of the mutant DSG4 in the ER induces ER stress, and the subsequent UPR may be a cofactor in the pathogenesis of monilethrix.

DISCUSSION

As the numbers of patients with monilethrix are limited, the pathogenesis of human *DSG4* mutations in the monilethrix is largely unknown. In this study, we identified a homozygous mutation, c.2119delG (p.Asp707Ilefs*109), in the *DSG4* gene from a Japanese patient. Although a few TEM analyses for moniliform hairs have been reported, the responsible genes are unknown (Gummer *et al.*, 1981; Ito *et al.*, 1984; Ito *et al.*, 1990). Despite the limited sample numbers in our study, the acantholysis of the cortex, which was seen in the lanceolate hair mouse (Kljuic *et al.*, 2003), was not observed. Instead, we observed abnormal keratinization in the cuticle and cortex of a moniliform hair (Figure 2c–f, Supplementary Figure S2B–S2E online) and fewer desmosomes in the patient's cuticle cells (Figures 2j and k, Supplementary Figure S2I–S2K online).

The keratinocyte intermediate filament network usually extends from the nuclear periphery toward the cytoplasmic membrane, where it interacts with desmosomal proteins. Keratin filament assembly alterations compromise hair integrity, and mutations in *KRT81*, *KRT83*, and *KRT86* cause monilethrix (Djabali *et al.*, 2003). Although KRT81, KRT83, and KRT86 are expressed in the middle/upper cortex, and in the lower and middle/upper medulla, DSG4 is expressed in a dynamic region of the hair bulb, where proliferation and differentiation intersect. Therefore, the mechanism of monilethrix by the *DSG4* mutation may differ from that of keratins.

To date, the molecular mechanism of aberrant DSG4-induced moniliform hair has largely been investigated in mouse models. Kljuic *et al.* (2003) reported the disturbed transition between proliferation and differentiation in the matrix of *Dsg4*-mutant mice, the lanceolate hair mice. They found abrupt differentiation in the lanceolate hair of *Dsg4*-mutant mice. Although some reports have been published

about human *DSG4* mutations (Kljuic *et al.*, 2003; Meyer *et al.*, 2004; Moss *et al.*, 2004; Rafiq *et al.*, 2004; Bazzi *et al.*, 2005; Messenger *et al.*, 2005; Bazzi *et al.*, 2006; Schaffer *et al.*, 2006; Shimomura *et al.*, 2006; Zlotogorski *et al.*, 2006; Farooq *et al.*, 2011), the pathogenesis is quite unclear. Similar to the mouse model, our TEM examinations revealed abnormal keratinization in the cortex and cuticle of the monilethrix hair. Moreover, fewer desmosomes were observed in cuticle cells. Our data provide details of human monilethrix due to the *DSG4* mutation.

Immunofluorescence studies using the mutant DSG4–specific antibody revealed a reduced but detectable level of mutant DSG4 expressed in the patient's hair cortex (Figure 3f), and the mutant *DSG4* mRNA seemed to be stable (Supplementary Figure S3 online). Our *in vitro* experiment revealed that the mutant DSG4 could not bind to PKG, and accumulated in the ER (Figures 4 and 5). Recently, it has been reported that keratinocytes from Darier disease display a constitutive ER stress response, concomitant with immature adherens junctions and desmosomes, resulting in decreased intercellular adhesion strength (Savignac *et al.*, 2014). ER stress induced by Ca^{2+} depletion reportedly controls differentiation through XBP1 activation in thapsigargin-treated normal keratinocytes (Celli *et al.*, 2011). Furthermore, UPR is activated in the suprabasal layer cells of normal human epidermis (Sugiura *et al.*, 2009). In our case, we clarified that ERAD occurred *in vitro,* by adding a proteasome inhibitor to the cells (Figure 6c). We also revealed that the mutant DSG4 caused chaperone induction in the patient's skin. Stronger GRP78/BiP expression was detected in the granular layer of the patient, as compared with healthy controls (Figure 6a and b). We also observed a significant reduction of the mutant DSG4 in the cortex, as compared with normal controls (Figure 3e), which would probably be due to a posttranslational event, such as ERAD.

Further investigations are necessary to clarify the involvement of ER stress in the pathogenesis of monilethrix. However, one of the possibilities is the downregulation of the desmosome due to the impaired transport of mutant DSG4 by the quality control mechanism in the ER. Indeed, distorted cuticle cells and fewer desmosomes were detected in the patient's hair by TEM analyses (Figure 2). The ER stress may also affect translational attenuation and compartments of the desmosome, such as desmoplakin, DSG, desmocollin, and E-cadherin. In addition, the TEM analysis of the constricted part of the moniliform hair revealed a dyskeratotic cortex and cuticle (Figure 2 and Supplementary Figure S2 online). Moreover, cytological abnormalities of cortical cells were observed. The accumulated mutant DSG4 may induce ER stress, and the damaged cells may undergo apoptosis. In Darier disease, rounded keratinocytes, corps ronds, are considered to correspond to apoptotic cells, which may result from an uncontrolled ER stress response (Savignac *et al.*, 2014). It is possible that the cytological abnormalities of the cortical cells detected in our TEM study may result from ER stress–induced apoptosis. Finally, abnormal keratinization can result from an abrupt transition from proliferation to differentiation (Kljuic *et al.*, 2003), and ER stress may induce some transcription factors that mediate such a transition. Ichthyosis follicularis with atrichia and photophobia syndrome, caused by deficiency in MBTPS2, is also thought to be due to ER stress (Oeffner *et al.*, 2009). It is possible that some other types of genetic hair loss may be caused in a similar manner to the hair loss elicited by the DSG4 mutations.

In conclusion, this work has described the details of moniliform hair caused by DSG4 through immunohistochemical and TEM analyses. Although the mechanism of monilethrix formation is still unknown, our data indicate that the mutant DSG4 may cause ER stress, which in turn exacerbates the abrupt transition from proliferation to differentiation of cells in the cortex. Management of ER stress may be a treatment modality for genetic hair diseases, such as monilethrix. Further cases and experiments are necessary to confirm the correlation between monilethrix and ER stress.

MATERIALS AND METHODS

Source of DNA

After written informed consent in accordance with the Declaration of Helsinki Principles protocols and approval from the ethics commission of Gunma University were obtained, peripheral leukocyte DNAs from the patient and her family, and from 50 unrelated healthy Japanese individuals, were prepared using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI).

Electron microscopy of the patient's follicles

Skin biopsies were taken from the patient's hair. The methods are described in detail in Supplementary Materials and Methods online.

Genetic analysis of the mutation site

All exons and exon–intron boundaries of *DSG4, KRT81, KRT83,* and *KRT86* were amplified by PCR, as described (van Steensel *et al.*, 2005; Shimomura *et al.*, 2006), and the amplified PCR products were directly sequenced.

Plasmid construction

The Wt DSG4 expression vector pCXN2.1-DSG4-Wt and the PKG expression vector pCMV-Tag2-FLAG-PKG were prepared as described previously (Farooq *et al.*, 2011). The pcDNA3.1-DSG4-Wt-HA, pcDNA3.1-DSG4-mut-HA, and pcDNA3.1-FLAG-PKG were generated as described in Supplementary Materials and Methods online.

Glycosidase digestion analyses

For the glycosidase digestion analysis, 10 μg samples were reacted with 1 μl of endoglycosidase H or PNGase F (New England BioLabs, Ipswich, MA), according to the manufacturer's instructions. Proteasome inhibition was achieved by treatment with 10 μM of MG132 (Peptide Institute, Osaka, Japan) for 4 hours at 37 °C.

Detection of DSG4 in the hair

Plucked hair proteins were extracted in NP-40/tris-buffered saline buffer. The proteins were detected by immunoblotting using a sheep polyclonal anti-DSG4 antibody (R&D Systems, Minneapolis, MN) and a rabbit polyclonal mutant DSG4–specific antibody. The methods are described in detail in Supplementary Materials and Methods online.

Immunofluorescence and immunohistochemistry studies

Samples were stained using a guinea pig polyclonal anti-DSG4 antibody (Acris Antibodies, San Diego, CA) and a mouse monoclonal anti-trichohyalin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). To detect mutant DSG4, the samples were stained using a rabbit polyclonal mutant DSG4–specific antibody. To analyze the intracellular localization of transiently expressed DSG4-Wt-HA and DSG4-mut-HA, HEK293T cells were used. The samples were reacted with a rat monoclonal anti-HA 3F10 antibody (Roche, Indianapolis, IN) and a rabbit monoclonal anti-calnexin antibody (Cell Signaling Technology, Denvers, MA). Samples were viewed with a confocal microscope (Olympus, FLUOVIEW FV10i). Immunohistochemical analysis was performed on formalin-fixed paraffin-embedded tissue specimens. Sections were incubated with an anti-GRP78/BiP antibody (Cell Signaling Technology). The methods are described in detail in Supplementary Materials and Methods online.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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